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Surveillance of SARS-CoV-2 RNA in wastewater: Methods optimization and quality control are crucial for generating reliable public health information

Warish Ahmed[1,a], Aaron Bivins[2,3,a], Paul M. Bertsch[1], Kyle Bibby[2], Phil M. Choi[4], Kata Farkas[5], Pradip Gyawali[6], Kerry A. Hamilton[7], Eiji Haramoto[8], Masaaki Kitajima[9], Stuart L. Simpson[10], Sarmila Tandukar[8], Kevin V. Thomas[4] and Jochen F. Mueller[4]

Abstract

Monitoring for SARS-CoV-2 RNA in wastewater through the process of wastewater-based epidemiology provides an additional surveillance tool, contributing to community-level screening and prevention efforts as these measurements have preceded disease cases in some instances. Numerous detections of SARS-CoV-2 RNA have been reported globally using various methods, demonstrating the technical feasibility of routine monitoring. However, to reliably interpret data produced from these efforts for informing public health interventions, additional quality control information and standardization in sampling design, sample processing, and data interpretation and reporting are needed. This review summarizes published studies of SARS-CoV-2 RNA detection in wastewater as well as available information regarding sample concentration, extraction, and detection methods. The review highlights areas for potential standardization including considerations related to sampling time and frequency relative to peak fecal loading times; inclusion of appropriate information on sample collection points; sample volume collected; transport and storage conditions; sample concentration procedures; RNA extraction process and performance; effective sample volumes; recovery efficiency testing; PCR inhibition; process controls throughout sample collection and processing; and PCR standard curve performance. Researchers are advised to follow the Minimum Information for Publication of Quantitative Real-Time PCR guidelines. Adhering to these recommendations will enable robust interpretation of wastewater monitoring results and improved inferences regarding the relationship between monitoring results and disease cases.

Addresses

[1] CSIRO Land and Water, Ecosciences Precinct, 41 Boggo Road, Dutton Park, QLD, 4102, Australia
[2] Department of Civil & Environmental Engineering & Earth Sciences, University of Notre Dame, 156 Fitzpatrick Hall, Notre Dame, IN, 46656, USA
[3] Environmental Change Initiative, University of Notre Dame, 721 Flanner Hall, Notre Dame, IN, 46656, USA
[4] Queensland Alliance for Environmental Health Sciences, The University of Queensland, Woolloongabba, QLD, 4102, Australia
[5] School of Natural Sciences, Bangor University, Deiniol Road, Bangor, Gwynedd, UK
[6] Institute of Environmental Science and Research Ltd (ESR), Porirua, 5240, New Zealand
[7] The Biodesign Institute Center for Environmental Health Engineering, Arizona State University, Temple, AZ, 85287, USA
[8] Interdisciplinary Center for River Basin Environment, University of Yamanashi, 4-3-11 Takeda, Kofu, Yamanashi, 400-8511, Japan
[9] Division of Environmental Engineering, Faculty of Engineering, Hokkaido University, North West 8, Kita-ku, Sapporo, Hokkaido, 060-0032, Japan
[10] CSIRO Land and Water, Lucas Heights, NSW, 2234, Australia

Corresponding author: Ahmed, Warish (Warish.Ahmed@csiro.au)
[a] Warish Ahmed and Aaron Bivins equally contributed to the conceptualization of the manuscript.

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Introduction

The current global pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is a public health emergency of international concern [1]. This pandemic has resulted in >40 million cases of coronavirus disease (COVID-19) and >1,120,000 deaths worldwide as of 22nd October 2020 [1,2]. Non-pharmaceutical public health interventions including clinical testing, social distancing, contact tracing, isolation of infected individuals, and in extreme epidemics, complete restriction of human mobility in areas ranging in size from town-suburb to state or province have been adapted to slow down the spread of the community transmission [3–5]. Although SARS-CoV-2 is

Table 1

Prevalence and concentrations of SARS-CoV-2 RNA in wastewater.

Country	Types of wastewater	Volume of wastewater concentrated (mL)	Sample storage before testing	Virus concentration methods used	Inclusion of process control	PCR inhibition checked	% recovery	RT-PCR assay/target gene used	Number of samples positive/number of samples tested (%) (concentration)	Sequencing	Reference
Australia	Untreated wastewater	100-200 (grab and 24-h composite)	Ice, 4 °C	Adsorption-extraction and ultrafiltration	No	Yes	NR	N_Sarbeco NIID_ 2019-nCOV	2/9 (22%) (1.9–12 GC/100 mL)	Sanger Illumina MiSeq	[9]
Brazil	Untreated wastewater	NR (10-h composite)	NR	Ultracentrifugation	No	No	NR	CDC N2	5/12 (41.6%)	NU	[42]
China	Inlets of pre-processing disinfection pool Outlet of pre-processing disinfection pool Final outlet of sewage disinfection pool	NR (grab)	NR	NR	No	No	NR	SARS-Cov-2 nucleic acid detection kit	3/3 (100%) 1/1 (100%) 0/1 (0%)	NU	[43]
China	Hospital septic tank influent Hospital septic tank effluent	2000 (grab)	NR	PEG precipitation	No	No	NR	CCDC-ORF1 CCDC-N	0/4 (0%) (500–1870 GC/L) 7/9 (78%)	NU	[44]
Czech Republic	Untreated wastewater	500 (mostly 24-h time or flow composite)	5 °C	Direct flocculation	Yes	Yes	35.5 ± 13.0% using TGEV (whole process control)	EliGene® COVID19 BASIC A RT kit	13/112 (11.6%)	NU	[45]
Germany	Untreated wastewater Treated effluent	45 (24-h flow composite samples)	Ice	Ultrafiltration and centrifugation	No	No	NR	M gene RdRP gene M gene RdRP gene	9/9 (100) (<10 GC/mL) 9/9 (100) (<100 GC/mL) 4/4 (100) (<10 GC/mL) 4/4 (100) (<100 GC/mL)	Sanger	[46]
India	Untreated wastewater Final effluents	50 (grab, 11:30am)	4 °C	Centrifugation, filtration, PEG precipitation	No	Yes	NR	TaqPath COVID-19 Combo Kit ORF1ab, N and S genes)	2/2 (100%) (560–350 GC/L 0/2 (0%)	NU	[36]
Italy	Untreated wastewater	250 (24-h composite)	–20 °C	Two-phase (PEG-dextran method) separation	No	No	NR	ORF1ab Spike protein RdRP	6/12 (50%) 2/12 (16.7%) NR	Direct sequence	[11]
Italy	Untreated wastewater	250 (24-h composite)	–20 °C	Two-phase (PEG-dextran method) separation	Yes	Yes	2.04 ± 0.70% using Alphacoronavirus HcoV-229E)	ORF1ab E gene RdRP	18/40 (45%) – Nested RT-PCR 26/40 (65%) (290–56,000 GC/L) – RT-qPCR NR	Direct sequence	[16]
Italy	Untreated wastewater Treated wastewater	NR (grab, 1pm)	NR	Membrane filtration	Yes	Yes	NR	ORF1ab N gene E gene ORF1ab N gene E gene	4/8 (50%) 0/4 (0%)	Whole genome sequencing	[47]

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Table 1. (*continued*)

Country	Types of wastewater	Volume of wastewater concentrated (mL)	Sample storage before testing	Virus concentration methods used	Inclusion of process control	PCR inhibition checked	% recovery	RT-PCR assay/target gene used	Number of samples positive/number of samples tested (%) (concentration)	Sequencing	Reference
Japan	Influent	200 (grab)	Ice	Electronegative membrane-vortex (EMV) method	Yes	Yes	71.6 ± 25.2% using MS2 for EMV 8.5 ± 3.7% using MS2 for the adsorption-extraction (RNA extraction and RT-qPCR)	N_Sarbeco NIID_2019-nCOV_N CDC N1 CDC N2	0/5 (0%) 1/5 (20%) (2400 GC/L)	Direct sequence	[10]
	Secondary treated wastewater	5000 (grab)		Adsorption–extraction							
Spain	Influent	200 (grab, 7–12 am)	4 °C	$Al(OH)_3$ adsorption–precipitation	Yes	No	10 ± 3.5% using PEDV (influent) 3.3 ± 1.6% using PEDV (effluent) 10 ± 2.1% using MgV (influent) 6.2 ± 1.0% (MgV effluent)	CDC N1, N2, N3 CDC N1, N2, N3 CDC N1, N2, N3	35/42 (83%) (5.10–5.50 log_{10} GC/L) 2/18 (11%) (5.40 log_{10} GC/L) 0/12 (0%)	NU	[13]
	Secondary										
	Tertiary effluent										
Spain	Untreated wastewater	200 (grab, 10 am–12 pm)	4 °C	$Al(OH)_3$ adsorption–precipitation	Yes	No	2.56–18.8% using MgV	CDC N1 CDC N2	12/15 (80%) (5.31–5.75 log_{10} GC/L) 13/15 (86.7%) (5.22–5.98 log_{10} GC/L)	NU	[48]
The Netherlands	Untreated wastewater	250 (24-h flow composite)	4 °C	Ultrafiltration	Yes	No	73 ± 50% using F-specific RNA phages for purification and concentration) 30.4 ± 22.3% using F-specific RNA phages for RNA extraction and RT-qPCR)	CDC N1 CDC N2 CDC N3 E_Sarbeco	18/29 (62.1%) (12–790 GC/mL) 18/29 (62.1%) (12–2200 GC/mL) 19/29(65.5%) (12–1800 GC/mL) 18/29 (62.1%)	NU	[12]
USA	Untreated wastewater	40 (24-h composite)	4 °C	PEG precipitation	No	No	NR	CDC N1 CDC N2 CDC N3	10/10 (100%) 10/10 (100%) (57–303 GC/mL) 10/10 (100%)	Sanger	[49]
USA	Untreated wastewater	100-750 (grabs at 7–11am and 24-h composite)	Ice, −80 °C	Centrifugation and ultrafiltration	Yes	No	54–56% using Phi 6	CDC N1 CDC N2	2/15 (13%) (3100 to −7500 GC/L)	NU	[14]
	Secondary treated effluent			Adsorption and elution with electronegative membrane							
	Final effluent										
USA	Sewage	125 (24-h flow composite)	Ice	InnovaPrep Electronegative filtration	Yes	Yes	5.5 ± 2.1% using BCoV) 7.6 ± 3.0 using BRSV) 4.8 ± 2.8% using BCoV) 6.6 ± 3.8 using BRSV)	CDC N1 CDC N2 CDC N3	107/198 (54%) 125/198 (65%) 113/198 (57%) (10–10,000 GC/100 mL)	NU	[50]
USA	Untreated wastewater	40–70 L (grab)	4 °C	NanoCeram electropositive cartridge	No	Yes	NR	CDC N1	54/54 (100%) (4–5 log_{10} GC/L)	NU	[51]

NR, not reported; NU, not undertaken; PEDV, porcine epidemic diarrhea virus; BCoV, bovine coronavirus; BRSV, bovine respiratory syncytial virus; TGEV, transmissible gastroenteritis virus; MgV, mengovirus.

Table 2

List of variables that may impact the detection of SARS-CoV-2 RNA in wastewater and accuracy of wastewater-based epidemiology.

Analytical steps	Variables	Examples	Key considerations
Wastewater sampling	Sampling method	Grab sample 24-h composite sample	Sampling time of day Autosampler sampling frequency Size of the sewer catchment Diurnal variation of fecal load HRT
	Sampling frequency	Hourly Multiple days per week Daily Weekly Bi-weekly	Available resources Autosampler Accessibility to the WWTP Co-operation from utilities and/or councils
	Sampling types/locations	Influent Primary effluent Secondary effluent Treated effluent	HRT Chlorine dose Suspended solids
Sample processing	Sample storage conditions	Refrigerated Frozen	Storage space Storage temperature
	Sample pretreatment	Pasteurization Prefiltration Centrifugation	Virus loss Time required for sample pretreatment
	Virus concentration methods	Adsorption–extraction Adsorption–elution PEG precipitation, Ultrafiltration, Ultracentrifugation	Virus recovery efficiency Effective volume analyzed Cost Speed
	Process control	Whole process control Molecular process control	Surrogate virus Same group of viruses Noninfectious Easy to source Easy to cultivate
Molecular detection	Viral RNA extraction	Direct extraction from membrane Extraction from the concentrated sample	Extraction efficiency Commercial extraction kit, In-house extraction method Manual extraction Robotic, extraction Cost Speed
	RT step	One step Two steps	cDNA synthesis kit, Enzyme RT primer Cost Efficacy
	PCR format	RT-PCR, Nested RT-PCR RT-qPCR RT-dPCR	Platform availability Sensitivity Speed Downstream analysis (e.g., sequencing)

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Table 2. (*continued*)

Analytical steps	Variables	Examples	Key considerations
	RT-PCR assay	CDC N1 CDC N2 E_Sarbeco N_Sarbeco NIID HKU CDC RdRP	Assay limit of detection Specificity Sensitivity Repeatability Intra and inter CV Duplexing or multiplexing
	PCR performance characteristics	R^2 value Efficiency Slope Y-intercept ALOD ALOQ	Inter CV Intra CV Repeatability Reproducibility Assay sensitivity
	Cut off Cq value	Cq value > 40	Fluorescence intensity threshold Inhibition
	Sequencing confirmation	Sanger sequencing Illumina MiSeq Direct sequencing Whole genome sequencing	Sequencing format PCR product amount Amplicon size Cost Cover
Data analysis	Reproducibility	Replication in sample processing Detection procedures	Reproducibility in biological and technical replicates PCR replicate (positive/negative)
	Adjustment of quantification data	Adjustment with process control recovery and/or virus recovery by concentration method	Reproducibility in biological and technical replicates PCR replicate (positive/negative)
	Analysis of left-censored data	Adjustment with process control recovery and/or virus recovery by concentration method	Adaptation of adjustment

HRT, hydraulic retention time.

predominantly a respiratory virus, SARS-CoV-2 RNA is also shed in feces (10^2–10^9 gene copies (GC)/mL) and urine (10^2–10^5 GC/mL) in addition to saliva and sputum (10^5–10^{11} GC/mL), which are often collected via wastewater systems [6,7]. Scientists around the globe are now trying to develop reliable approaches to monitor the virus circulation within communities by measuring SARS-CoV-2 RNA in wastewater by an approach known as wastewater-based epidemiology (WBE) [8]. Several recent studies have reported the presence of SARS-CoV-2 RNA in wastewater in several countries [9–14].

Lodder and Husman [15] reported detection of SARS-CoV-2 RNA in wastewater in the Netherlands within 4 days of the first clinically diagnosed should be case in the country. Detections of SARS-CoV-2 RNA in wastewater have been reported in Milan, Italy, within a few days of the first national case [16] and in Brisbane, Australia, when the number of clinical cases were in the hundreds within a population of approximately 600,000 [9]. Interestingly, Medema et al. [12] detected an RNA target associated with SARS-CoV-2 in wastewater from a city in the Netherlands 6 days before the first clinical cases were reported. These observations indicate that WBE could be a feasible and sensitive means of monitoring SARS-CoV-2 infection presence and trends within communities. A modelling exercise has suggested that wastewater surveillance could theoretically detect one SARS-CoV-2 infection among 2,000,000 individuals, but noted limitations including uncertainties around temperature-dependent RNA signal decay in wastewater and hydraulic residence times in wastewater collection systems before sample collection [17]. A preprint has reported a more modest detection limit of one fecal-shedding infection in 1000 to two in 10,000 as estimated from monitoring wastewater from a hospital with COVID-19 treatment and isolation units [18]. However, it should be noted that this estimation was not based on virus recovery corrections. Also, the authors extrapolated a single quantification cycle (Cq) value for a wastewater sample resulting from a known proportion of infections to the estimated threshold limit.

The sensitive detection of SARS-CoV-2 RNA in wastewater, and thereby the presence of infections within a community, depends on both the wastewater sampling and the molecular-based methods used. These methods remain diverse and unstandardized, and often lack important information needed by public health units to interpret and apply the information [19–21]. To date, little has been documented on the performance of concentration, extraction, and detection methods for SARS-CoV-2 in wastewater [22]. Few of the published SARS-CoV-2 WBE articles provide detailed experimental procedures, which hinders our ability to replicate the experiments or to compare across studies, as is necessary to improve interpretation of the results of WBE to inform public health officials. In this opinion paper, we discuss the peer-reviewed journal articles that have reported the presence of SARS-CoV-2 RNA in wastewater and provide recommendations to encourage better quality control, allowing for more reliable and less ambiguous interpretation and application of WBE results.

Published studies detecting and enumerating SARS-CoV-2 RNA in wastewater

Table 1 summarizes the peer-reviewed research on the prevalence and concentration of SARS-CoV-2 RNA in wastewater as observed in various countries. For untreated wastewater, sample volumes ranging from 40 to 500 mL are typically concentrated; however, for secondary- and tertiary-treated wastewater, larger sample volumes, up to 70 L, have been concentrated. Of the 18 studies, eight studies collected composite samples over durations ranging from 10 to 24 h, two studies collected both composite and grab samples, and eight studies collected grab samples at a single or a few time points. Various virus concentration methods, including polyethylene glycol (PEG) precipitation, adsorption–extraction and adsorption–elution using electronegative membranes, ultrafiltration, ultracentrifugation, Nano-Ceram electropositive cartridge, and direct flocculation, have been used to detect SARS-CoV-2 RNA in wastewater. The majority of the studies (11 of 18) used a single concentration method, whereas the others used two different concentration methods without evaluating each method's efficiency in concentrating enveloped SARS-CoV-2 from wastewater.

Of the 18 studies, nine studies used either a whole (concentration to RT-qPCR) process control or a molecular (RNA extraction to RT-qPCR) process control. Only eight of the studies tested wastewater samples for the presence of PCR inhibitors (that impede amplification) using a process control, however, very limited information has been provided with respect to magnitude and frequency of PCR inhibitors in wastewater samples. Ten studies did not provide any information on the method's recovery efficiency, and the remaining studies determined the recovery efficiency using a variety of enveloped (Phi 6 phage, porcine epidemic diarrhea virus, bovine coronavirus, bovine respiratory syncytial virus, and transmissible gastroenteritis virus) and nonenveloped (F-specific RNA phage, mengovirus) viruses. Also, a wide array of RT-qPCR assays was used to detect and enumerate molecular targets associated with the SARS-CoV-2 genome, often with little or no information provided on the RT-qPCR assay performance characteristics. All these factors influence the ability to compare results among studies. Several studies provided quantitative data on the numbers of GC/L of wastewater, whereas some studies only provided

positive/negative results. Only six studies confirmed the identity of SARS-CoV-2 RNA using sequencing approaches, and of these studies, none reported false-positive results suggesting RT-qPCR assays used are highly specific.

Recommendations for SARS-CoV-2 WBE

Although the published studies have assisted to establish the technical feasibility of routine monitoring for SARS-CoV-2 RNA in wastewater, it is apparent that there is generally a lack of quality control information in the published literature for WBE of COVID-19. This lack of reporting on quality control has the potential to limit the interpretation and usefulness of the produced data for advancing the WBE field, and ultimately implementing public health interventions. A list of variables that are likely to impact the sensitive and accurate detection/quantification of SARS-CoV-2 RNA in wastewaterfor public health surveillance are shown in Table 2. Increasing interest in WBE among public health officials and the introduction of state-wide or national monitoring programs in several countries demands improved reporting of methodological details and quality control metrics [23]. A recent review even suggested the need for an optimized and univocal methodological framework concerning the detection and quantification of SARS-CoV-2 RNA in wastewater [20]. Scaling SARS-CoV-2 wastewater surveillance to deliver national programs will also likely require that testing be performed not only by research laboratories but also by commercial laboratories. Such a rapid expansion in testing capacity makes robust and reproducible methods and quality control vital to produce actionable public health information. In view of this need, we recommend methodological and quality assurance approaches for SARS-CoV-2 RNA detection in wastewater using molecular methods.

Wastewater sampling

Sampling design is a pivotal factor for detecting SARS-CoV-2 RNA in wastewater. The concentration of SARS-CoV-2 RNA in influent wastewater is expected to vary diurnally, based on defecation frequency and timing, as well as the sampling technique and frequency. Defecation in the general population is most frequent in the early morning compared with other times [24]. Therefore, wastewater collected during periods of peak fecal loading may be more enriched in SARS-CoV-2 RNA than wastewater generated at other times in the day. In situations where an autosampler is not available, periods of peak fecal loading (if known) should be targeted for grab sampling. One or more grab samples taken during peak fecal loading would provide a higher probability of SARS-CoV-2 RNA detection. We recommend that local peak fecal loading times be identified priot to implementing a grab sampling campaign. Although the exact peak fecal loading periods will vary between wastewater treatment plants (WWTPs) due to differences in sewer infrastructure, total influent flow may be a useful proxy for anthropogenic activity and fecal shedding in the morning.

Although a toilet flush cycle lasts only for several seconds, the resultant pulse of wastewater disperses across time depending on wastewater collection system characteristics, such as pumping stations [25]. For example, flushes of anthropogenic gadolinium (used as an MRI contrast agent) in a WWTP catchment (approximately 100,000 inhabitants) were found to arrive at the WWTP in discrete pulses ranging from 4 to 20 min wide [26]. Compared with grab samples, composite wastewater samples collected with an autosampler are much better suited to adequately sampling these pulses. Where an autosampler is available, autosamplers should composite as frequently (e.g., 10–15 min) as possible. This is particularly important for sampling campaigns aiming to detect shedding by very few individuals. A flow-weighted composite sample is strongly recommended as it accounts for the often-numerous fluctuations in flow experienced at the inlet of a WWTP [26]. If this is not possible, a time-based composite sample is recommended. If an autosampler is unavailable, sampling during peak fecal loading is recommended as above. Details regarding autosampler setup and grab sampling time have been poorly reported to date (Table 1) and should be regularly reported to aid the interpretation of results.

Sampling frequency (i.e. number of discrete samples collected) is another important factor that needs to be considered for WBE of COVID-19 in the community. Although most adults defecate once every 24 h or less [24], studies published to date indicate that some infected people do not shed the virus consistently, if at all. Consequently, two or more 24-h composite samples per week, or one 48- or 72-h composite sample per week is ideal for sampling programs aiming to sample the majority of shedding events in a community. Depending on the resources available, weekly sampling is recommended as a minimum with twice-weekly sampling preferably on weekends providing increased resolution. Where sample analysis costs or resources are restrictive, pooling samples from adjacent catchments for analysis may be a useful, particularly in areas served by multiple small WWTPs or in scenarios where a positive detection is unlikely. For WWTP catchments with a significant transient population (e.g., day workers or weekend visitors), comparison of morning or afternoon samples, or weekend and weekday samples may provide some insight as to the movement pattern of the shedder(s). Information on sample volume collected, collection points (e.g., influent, before/after grit removal, primary clarifier), transport conditions, and inclusion of a field

blank must be clearly detailed for comparison between studies.

Wastewater sample storage and pretreatment

Following sample collection, storage conditions can also affect the detection of SARS-CoV-2 RNA signals in wastewater samples because of stability. It is recommended that samples should be transported on ice from the collection point to the laboratory. On arrival at the laboratory, samples should be stored at 4 °C and should be concentrated within 48−72 h. Several studies have reported prolonged persistence of enveloped viruses, including SARS-CoV-2 and the RNA in wastewater samples at ∼4 °C [27−30]. Therefore, short-term storage, for 1−5 days at 4 °C may be appropriate followed by concentration and extraction for RT-qPCR analysis. The impacts of storing untreated bulk wastewater samples at −20 and −80 °C are not known. Furthermore, the impacts of pasteurization and repeated freeze−thaw cycles on the degradation of SARS-CoV-2 RNA in wastewater are not well understood and should be avoided until more data become available. We acknowledge that pasteurization is often undertaken to minimize the risk associated with handling wastewater. However, when the concentration of SARS-CoV-2 RNA is expected to be low in wastewater, this approach is not recommended as it may produce false-negative results. We also encourage researchers to provide critical information on sample metadata such as biological oxygen demand, chemical oxygen demand, total suspended solid, pH, and, storage temperature, storage time before sample processing, whether the samples were frozen before virus concentration, as well as any pretreatment before the concentration step.

SARS-CoV-2 concentration

Several virus concentration methods have been developed for the detection of enteric viruses in water and wastewater matrices [31]. However, some of the approaches may not be suitable for concentrating enveloped viruses including SARS-CoV-2 from wastewater [7,19]. Virus concentration is particularly important because the concentration of SARS-CoV-2 in wastewater is expected to be low in the beginning or at the tail end of an epidemic. To provide an effective early warning system or to inform decisions on an easing of restrictions safely, the methods must be sensitive enough to detect a very small number (low concentration) of viruses in a wastewater sample.

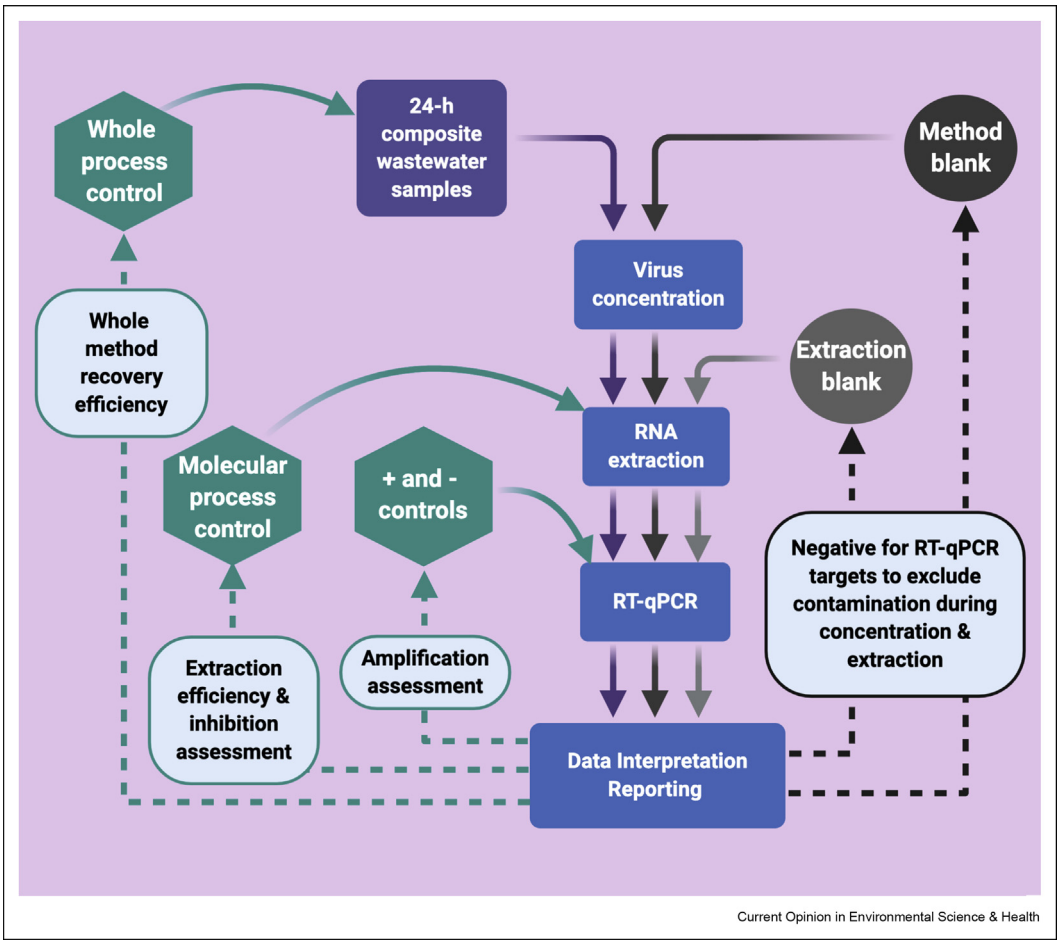
A recent study evaluated seven concentration methods for the recovery of murine hepatitis virus, a surrogate of SARS-CoV-2, in a small volume (e.g., 50 mL) of untreated wastewater. Among the methods tested, the electronegative membrane, with the addition of $MgCl_2$ resulted in the greatest mean recovery rate [32]. Such a method is suitable for several reasons: it does not involve any prefiltration and precentrifugation step, and viruses are adsorbed on the membrane from both the liquid and solid fractions of the wastewater. If a larger diameter membrane is used, up to 200 mL wastewater can be processed with this method. However, this method may not be suitable for highly turbid and medium-sized wastewater samples (500 mL−1 L). For wastewater volumes greater than 200 mL, the PEG precipitation method can be a suitable option [19]. Both adsorption–extraction and PEG precipitation methods can be performed in most laboratories with basic equipment and do not require extensive technical expertise. Further studies need to be conducted and validated to better understand the difference in recovery between enveloped and nonenveloped viruses using various concentration methods. We also recommend including a method blank (analyte free matrix) for each batch of wastewater samples processed to assess cross contamination from sample analysis. A flow chart showing steps and quality control measures that should be taken for reliable and comparable data from WBE for SARS-CoV-2 are presented in Figure 1.

PCR inhibition and process control

PCR-based assays are prone to the inhibitors that can be found in wastewater, which affect the sensitivity of the assay and may result in false-negative results [33]. Wastewater influent compositions are highly variable within a single WWTP and the variability in composition is even greater between WWTPs. Wastewater samples contain polysaccharides, metal ions, and RNases, which can inhibit RT-PCR amplification [33,34]. These PCR inhibitors may be co-concentrated during virus concentration for some methods. Therefore, the presence of inhibitors in wastewater samples needs to be investigated, and if present, inhibition should be both reported, and efforts should be taken to minimize overall inhibition. To achieve this, we recommend that each wastewater sample should be seeded with a surrogate virus as a whole-process control to obtain information on the surrogate virus recovery and RNA recovery and RT-PCR inhibition for the entire process starting from sample concentration to RT-PCR detection [31]. If an appropriate process control is not available, molecular process controls can also be included to obtain information on the RNA extraction efficiency, and RT-PCR inhibition should, at a minimum, be evaluated (e.g., through target dilution) and reported. Based on the process control data, samples may need to be reanalyzed (e.g., after dilution to reduce PCR inhibitors to suitable levels) or switch to an alternative method for increased sensitivity [31].

For a reliable process control, it is appropriate to select a virus that is morphologically and genetically similar to the target virus and is expected not to be present in the tested water. For example, for SARS-CoV-2, low-pathogenic animal CoVs such as murine hepatitis virus, bovine

Figure 1



A flow chart showing steps and quality controls for producing reliable and comparable information of SARS-CoV-2 RNA for wastewater-based epidemiology.

coronavirus, feline infectious peritonitis virus or enveloped bacteriophages, such as Phi 6 phage, represent ideal controls. We acknowledge that obtaining a suitable process control may be difficult for many laboratories, especially during a pandemic. Nonetheless, the selection of already established process controls for enteric viruses, such as single-stranded murine norovirus and mengovirus, may be preferable to include as a process control [35]. Viruses that are already present in wastewater in high numbers, such as pepper mild mottle virus or F-specific RNA coliphage phage may be used as a process control as long as their concentrations in the original and in the concentrated samples are compared to assess recovery levels. Nonetheless, the structure and size of these viruses are substantially different from the structure of SARS-CoV-2; therefore, they may provide an inaccurate estimation of recovery. However, these viruses can be used as a qualitative control to check the successful extraction of RNA from wastewater samples.

RNA extraction

To obtain high-quality RNA of SARS-CoV-2 for RT-qPCR analysis, several factors need to be considered, including the RNA extraction procedures, the concentration, purity, and integrity of the extracted RNA, risks of cross-contamination by DNA/RNA that may be present in the laboratory (equipment, surfaces, air), or the presence of other contaminants. Several studies have used various commercially available RNA extraction kits for the extraction of SARS-CoV-2 RNA from wastewater. The recovery of RNA may vary greatly depending on the kits used, and the performance of kits may vary considerably between manufacturers. The majority of studies used 140–450 μL of concentrated samples for RNA extraction and obtained 30–100 μL of viral RNA extracts [9,10,12,13,36]. The volume of concentrated samples used for the viral RNA extraction step and the resulting volume of RNA extracts may also need to be optimized to reduce the inhibition during the

downstream RT-qPCR analysis that also influence the probability of detecting SARS-CoV-2 RNA. Little has been documented on the performance of various commercial RNA extraction kits for the recovery of SARS-CoV-2 from concentrated wastewater samples. In our opinion, RNA extraction kits that have fewer steps and are equipped with PCR inhibitor removal techniques are likely to be more useful to reduce the chance of contamination and downstream inhibition. Both virus concentration and RNA extraction methods need to be equally effective for the isolation of low levels of SARS-CoV-2 from wastewater. We also recommend including a reagent blank (negative extraction control) for each batch of RNA extraction to document the absence of cross-contamination from reagents during the extractions.

RT-qPCR/dPCR QA/QC

For detection and quantification of SARS-CoV-2 RNA in wastewater, RT-PCR, RT-qPCR, and RT-digital PCR (dPCR) have been used. These PCR technologies use different platforms, reagents, protocols, analysis methods, and reporting formats, which results in a lack of methodological consistency in PCR experiments and the resulting data. Bustin et al. [37] and Huggett et al. [38], recommended minimum information for publication of both qPCR and digital PCR experiments to ensure the experiment's accuracy, correct interpretation, and repeatability. To generate data of the highest quality, WBE researchers should adhere to the published Minimum Information for Publication of Quantitative Real-time PCR (MIQE) and the Digital MIQE guidelines as strictly as possible. All PCR experiments should include the appropriate no-template controls and positive controls (for RT-PCR) and standards (for RT-qPCR). Standard curves can be prepared from a diverse array of materials including synthetic DNA or RNA of the amplicon, plasmid constructs, cDNA cloned into plasmids, and RNA extracted from the biological samples [37]. Each of these standards materials confers certain advantages and disadvantages [39]. Therefore, it is vital that the type of standard material used is fully described including the manufacturer and method of determining the copy number for use in calibration. In addition, any required treatment, such as digestion or linearization of circular control plasmids, should be reported as circular plasmids have been observed to cause quantification bias [40]. In the case of RT-qPCR experiments, standard curve characteristics (slope, y-intercept, r^2 value), Cq values, and estimated copy numbers should be reported. A fresh diluted standard curve should be used when a Cq shift of 0.5−1.0 is observed [37]. The standard curve should be used in every RT-qPCR run if possible. Alternatively, a master standard curve compiled from multiple independent experiments can also be used [41]. For digital PCR experiments, the metrics required to calculate the most probable copy number (total number of partitions and number of positive partitions, partition volume) should be reported. We also recommend that PCR experiments include technical replicates for each sample and SARS-CoV-2 specific targets. For each SARS-CoV-2 specific assay, assay limits of detection should be reported, including the method of determining such limits. Ideally, the specificity of the assays used should be confirmed by sequencing or analysis of the resulting PCR amplicons.

Reporting turnaround time

Although wastewater surveillance for SARS-CoV-2 has the potential to act as an early warning system, the merit for WBE will be influenced by site selection and can only be realized with rapid turnaround. The value of results from wastewater surveillance decreases with increasing turnaround time, particularly in cases where other methods do not provide rapid, objective information. To maximize this value, sampling programs should aim to minimize the time taken between the stages of approvals, wastewater sampling, analysis, reporting, and consequent action based on the results. We recommend that each program develop its own unique operating protocols for each of these four aspects to maximize the value of surveillance efforts. Sites may be selected for many reasons based on the status of the pandemic in a particular region. The fast commencing of sampling will require an efficient approval process and close collaboration with those responsible for sewer networks, such as city councils, water utilities, and service providers. If WBE is being applied to small populations, as may be the case with age care facilities, prisons, airline, cruiseships, and university campus accommodation, ethics approvals may be needed. Rapid response coupled with rapid turnaround clearly provides the best chance of capturingSARS-CoV-2 RNA detection and intervention in the community.

Concluding remark

WBE has been shown to be a powerful and effective tool to assess viral infections at a community level. Extraordinary efforts have been made globally to investigate SARS-CoV-2 in wastewater as well; however, to date, there is no identified gold standard method for the concentration, extraction, and detection of the virus in complex environmental matrices, such as sewage. To obtain accurate results, sampling, sample process, and viral quantification methods should be evaluated and validated. Samples should be taken on a regular basis and transported chilled to laboratories where they should be stored at 4 °C and processed within 2−3 days. Several sample concentration protocols are available and may be useful for SARS-CoV-2 recovery; however, their performance may vary among samples and hence appropriate process controls should be used. Quantitative and digital PCR methods have been shown to

detect SARS-CoV-2; however, these methods may be affected by inhibitors. Efforts should be taken to reduce the amount of inhibitors during RNA extraction and appropriate controls should be used to assess false-negative and positive readings. Following these guidelines, actionable and reliable SARS-CoV-2 RNA concentrations in wastewater can be obtained. Results can then be compared and further evaluated on an international level to assist the mitigation of the pandemic.

Credit author statement

Warish Ahmed: Conceptualization, Writing - original draft, Aaron Bivins: Conceptualization, Writing - original draft, Paul M. Bertsch: Writing - original draft, Kyle Bibby: Writing - original draft, Phil M. Choi: Writing - original draft, Kata Farkas: Writing - original draft, Pradip Gyawali: Writing - original draft, Kerry A. Hamilton: Writing - original draft, Eiji Haramoto: Writing - original draft, Masaaki Kitajima: Writing - original draft, Stuart L. Simpson: Writing - original draft, Sarmila Tandukar: Writing - original draft, Kevin Thomas — Writing - original draft, Jochen Mueller: Writing - original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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